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**Variants in the *LGALS9* Gene Are Associated With Development of Liver Disease in Heavy Consumers of Alcohol.**

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# GENETIC VARIATION IN *LGALS9* CONFERS SUSCEPTIBILITY TO ALCOHOLIC LIVER DISEASE

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**Short title: GAL9 SNPS in ALD**

**Abbreviations:** **Acon** Alcoholic Controls; **ALD** Alcoholic Liver Disease; **Gal-9** Galectin-9; **MAF** Minor allelic frequency; **SNPs** Single Nucleotide Polymorphisms

**Contributions:** Hugo R. Rosen: Designed the study, interpreted data and wrote the manuscript. Lucy Golden-Mason: Was involved in the acquisition, analysis and interpretation of data and drafting of the manuscript. Ivana Yang: Participated in data acquisition and analysis. Ann K. Daly: Contributed to interpretation of data and critical review of the manuscript. Christopher P. Day: Contributed to interpretation of data and critical review of the manuscript.

**Abstract:**

**Background & Aims:** Alcohol consumption is a major cause of chronic liver disease, accounting for a large proportion of cirrhosis-related deaths worldwide. Only a fraction of heavy alcohol drinkers develop advanced alcoholic liver disease (ALD), indicating that other factors likely contribute to the phenotypic heterogeneity. Herein, we explored whether polymorphisms in the gene encoding Galectin-9 (*LGALS9*), previously shown to play key roles in mediating liver injury, were associated with development of ALD. **Methods:** The study consisted of 554 individuals with at-risk alcohol consumption in the absence of other risk factors for chronic liver disease; 375 patients had developed ALD. All subjects were white Europeans who had consumed more than 80g of ethanol per day; 179 of these were controls with normal liver function and no clinical evidence of liver disease. **Results:** Using data from the HapMap project, 5 single nucleotide polymorphisms (SNPs) that tag all the common haplotypes were identified. Four of these SNPs (rs3751093, rs4239242, rs732222 and rs4794976) were associated with increased risk of developing ALD. Moreover, using cells from healthy subjects, we found that the transcript and protein levels of Galectin-9 varied according to the carrier state of these alleles. Multivariate analysis confirmed that rs4239242 and rs4794976 were associated with risk of ALD. **Conclusion:** The sequence variations reported here may provide predictive information regarding the risk of developing ALD in at-risk individuals and identify novel therapeutic targets for this common disease.

**Keywords:** Genetics; Cirrhosis; Human

Word count: 3435

Alcohol consumption is a major cause of chronic liver disease, accounting for a large proportion of cirrhosis-related deaths worldwide <sup>1</sup>. Alcoholic liver disease (ALD) comprises a broad spectrum of liver injury, ranging from simple steatosis to more severe forms, including alcoholic hepatitis, cirrhosis and hepatocellular carcinoma. Less than a third of heavy alcohol drinkers develop advanced ALD, indicating that other factors likely contribute to the phenotypic heterogeneity; these factors include gender, obesity, drinking patterns and duration, and non-sex-linked genetic factors <sup>1, 2</sup>. In this regard, genetic variation in the patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene has been associated with an increased risk of both alcohol-related cirrhosis and cancer<sup>3-6</sup>. Candidate gene case-control studies have also found associations between ALD and single-nucleotide polymorphisms (SNPs) within genes coding for alcohol-metabolizing enzymes, antioxidant enzymes, and cytokines <sup>7</sup>. Excessive tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production is characteristic of ALD. Two TNF- $\alpha$  SNPs have been associated with the progression of ALD, although results in subsequent studies have been discrepant <sup>7</sup>.

Galectins are evolutionarily conserved glycan-binding proteins with wide tissue distribution and critical functions in immune tolerance and inflammation <sup>8</sup>. Emerging data has implicated Galectin-9 (Gal-9), a tandem repeat-type galectin with two carbohydrate recognition domains, in hepatic immune homeostasis <sup>9</sup>. We had first reported that Gal-9 was upregulated by Kupffer cells in patients with chronic HCV infection, induced apoptosis of viral-specific CD8<sup>+</sup> T cells, and that it increased production of pro-inflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) in liver-derived and peripheral mononuclear cells <sup>10</sup>, implicating Gal-9 as a key mediator in pathways that lead to liver

injury. Subsequent reports have ascribed central roles for Gal-9 in HBV-related hepatocellular carcinoma <sup>11</sup>, autoimmune hepatitis <sup>12</sup>, and ischemic-reperfusion injury in the liver <sup>13</sup>. Based on the broad functions of Gal-9 in a wide range of hepatic disorders, we hypothesized that functional genetic polymorphisms in the gene coding for Galectin-9 (*LGALS9*) would be associated with development of ALD.

## **PATIENTS AND METHODS**

### **Study subjects:**

To be included in the study patients had to have been consuming more than 80 g ethanol/day for at least 10 years at the time of presentation, as detailed previously<sup>14-16</sup>. After obtaining informed consent, blood (10 ml) was collected. All patients were Caucasian and originated in the north-east of England as did their parents and grandparents. Heavy drinkers were recruited from two sources: referrals to the hospital liver unit with suspected ALD; and referrals to the Regional Alcohol Addiction Unit with alcohol dependency. Patients who presented to the liver unit had their detailed lifetime alcohol history taken by a specific alcohol research nurse, and those who presented to the Alcohol Addiction Unit by a community psychiatric nurse trained in drug and alcohol addiction<sup>14</sup>. The presence and severity of ALD was determined initially by standard liver blood tests. Patients with either alanine transaminase, alkaline phosphatase, or bilirubin more than twice the upper limit of normal on two occasions within a six month period were further investigated with ultrasonography. If there was no evidence of biliary obstruction, liver biopsy was performed unless contraindicated by coagulopathy (prothrombin time more than three seconds prolonged). The criteria for inclusion into the ALD group were either liver histology compatible with ALD or clinical evidence of hepatic decompensation. For inclusion into the Alcoholic Control (Acon) group patients had to be actively drinking, have no clinical evidence of liver disease, and have either: normal liver blood tests on two occasions (not including an isolated rise in  $\gamma$ -glutamyl transferase) or, for those with abnormal liver blood tests, liver histology showing either normal liver or steatosis with no evidence of steatohepatitis or fibrosis. Exclusion

criteria for this study were as follows: positivity to hepatitis B virus surface antigen or antibody to hepatitis C virus; other type of liver disease, including primary biliary cirrhosis, autoimmune hepatitis, primary sclerosing cholangitis, hemochromatosis (and other metabolic diseases), or alpha1-antitrypsin deficiency.

In addition, 34 healthy subjects with no significant alcohol history were used to study the effect of PBMC stimulation on Gal-9 transcription in order to determine if SNPs were associated with differences in transcript and protein expression.

### **SNP analysis:**

The selection of a candidate gene for an association study is usually based on the biological plausibility in chosen gene(s) that play a putative role in the pathogenesis of the disease of interest. The *LGALS9* gene is located on human chromosome 17 (Chr17:25,958,174-25,976,586). Using data from the HapMap project, 5 SNPs that tag all the common haplotypes (frequency > 0.01) were identified using Haploview. Genomic DNA was isolated from peripheral blood mononuclear cells (PBMC) from each of the study subjects. Genotyping for the selected *LGALS9* polymorphisms was performed using allelic discrimination by LGC Genomics, Herts, UK. In order to determine whether the allelic frequencies varied according to ethnicity, we used the 1000 Genomes Project (<http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes>). The average frequencies were determined from multiple groups: for whites (GBR- British in England and Scotland, FIN- Finnish in Finland, IBS – Iberian population in Spain, TSI-

Toscani in Italia, CEU – Utah residents with Northern and Western European ancestry); Hispanics (CLM- Columbians from Medellin, Columbia, MXL- Mexican Ancestry from Los Angeles, PUR- Puerto Ricans from Puerto Rico); for African Americans (ASW- Americans of African ancestry in SW USA).

### **In vitro assays:**

PBMCs from control individuals who did not consume significant alcohol (n=34), at a concentration of 1 million/ml in 24 well plates, were cultured with IFN- $\gamma$  (25ng/ml; R&D Systems), ethanol (EtOH, 25nM; Sigma) or a combination of both (IFN/EtOH) for 24 hours. Cells were harvested; RNA isolated using the RNeasy Mini kit (Qiagen), quantified using a Nanodrop microspectrometer and 1  $\mu$ g of RNA transcribed to cDNA using the Quantitect RT kit (Qiagen). Expression of *LGALS9* was assessed by the Step One Plus Real time PCR system using the Fast SYBR Green Master Mix Protocol (Applied Biosystems). QuantiTect primer assays for use with Sybr Green were purchased from Qiagen/Superarray. For 8 selected individuals, cells were cultured as above and harvested for analysis of cell surface Gal-9 expression by monocyte subsets (% positive compared to isotype control) using flow cytometry. Acquisition was performed using a BD FACSCanto II instrument (BD Biosciences, San Jose, CA) compensated with single fluorochromes and data was analyzed using Diva™ software (BD Biosciences). Monocytes were distinguished by positive staining for CD14 and/or CD16 and high levels of HLA-DR, within a low forward-side scatter gate. Monocyte subsets were distinguished by the presence or absence of CD16 expression. HLA-DR FITC clone LN3 was from eBioscience, CD14 APC-Cy7 clone M $\phi$ P9 was from BD



PharMingen. Biolegend antibodies used were: CD16 pacific blue clone 3G8, Galectin-9 PE clone 9M1-3, and IgG PE clone MOPC-21.

### **Statistical analyses:**

PHASE software (v.2.1.1) was used for reconstructing haplotypes from genotype data using Bayesian statistics (see <http://stephenslab.uchicago.edu/software.html>)<sup>17</sup> Fisher's exact test and Bonferroni-corrected threshold were used for significance ( $p < 0.05$ ). Odds ratio and its 95% confidence interval were calculated for genotypes in ALD and control groups. The Wilcoxon signed-rank test was used to compare fold increases of stimulated conditions with control conditions, and the non-parametric Mann-Whitney U-test was used to compare differences between groups. Calculations were performed using GraphPad Prism software.

## RESULTS

A total of 554 individuals with at-risk alcohol consumption in the absence of other risk factors for chronic liver disease comprised the current analyses. All subjects were white Europeans who had consumed more than 80g of ethanol per day for 10 or more years. Of these, 375 patients had ALD, including 268 (71%) with cirrhosis and 74 (19.7%) with alcoholic hepatitis. As alcohol controls (Acon), 179 subjects with similar duration and degree of alcohol consumption but no clinical evidence of liver disease and normal liver function tests on at least two occasions (isolated increase in gamma-glutamyltranspeptidase acceptable) were used. This latter Acon group had a median age of 42 years, compared to 49 years for the ALD group and 73% were male, compared to 71% in the ALD group.

### **Variants in *LGALS9* associated with development of ALD in at-risk individuals**

Genomic DNA was isolated from this highly homogeneous group of 554 individuals with similar exposures to alcohol but distinct phenotypic outcomes. As described in the Methods, we tested five sequence variants in the human *LGALS9* gene. Four of these SNPs were associated with the variable risk of developing ALD (**Table 1**). The frequencies of homozygous carrier state of the rs732222 [G] was over-represented in the group not developing ALD. For the *LGALS9* rs3751093, the proportion of homozygote G-allele carriers was significantly higher among at-risk individuals who were protected from developing alcohol-liver injury as compared to A-allele carriers who were more prone to develop ALD. We also found an association

between the rs4239242 [T] allele and protection from ALD; the presence of the C allele, as homozygote or heterozygote was associated with a 70% difference in risk of developing ALD (OR 1.72, CI: 1.21-2.46). The frequencies of homozygous carrier state of the rs4794976 [T] was over-represented in the group not developing ALD. In contrast, we did not find an association with the rs3763959 variant and risk of ALD. Haplotypes represent a combination of alleles at different markers along the same chromosome that are inherited as a unit <sup>18, 19</sup>. The distribution of haplotypes inferred for the variants are shown (**Table 2**); the H3 haplotype was protective against ALD ( $p = 0.016$ ) whereas the H4 haplotype was more frequent in patients with ALD ( $p = 0.024$ ), although the numbers are relatively small.

### **Functional genomics: SNPs associated with variable production of Gal-9**

As these data are potentially consistent with loss-of-function and gain-of-function alleles, we next tested whether the natural occurrence of these variants was associated with expression of Gal-9 following stimulation with either IFN- $\gamma$  (known to be a stimulant of Gal-9) <sup>10</sup>, ethanol (25 nM) or the combination. PBMC from thirty-four healthy subjects with no significant alcohol history were stimulated in culture for 24 hrs. Interestingly, *LGALS9* rs732222 GG homozygotes, rs3751093 GG homozygotes, and rs4239242 TT homozygotes demonstrated lower levels of Gal-9 transcription with ethanol stimulation (**Figure 1**), potentially consistent with genotype-associated protection from ALD. Next, we selected normal subjects with “protective” SNPs (over represented in the Acon group) and those with “detrimental” variants (over-represented in the ALD group), as shown in Supplemental Table 1. We stimulated cells with ethanol, IFN- $\gamma$  (a known

stimulant of Gal-9 and an inducer of liver injury in rodent models of ALD<sup>20</sup>), either individually or in combination. Using a FACS-based staining assay, we demonstrated that total monocytes as well as CD14<sup>pos</sup>CD16<sup>neg</sup> classical monocytes <sup>21</sup> (the more frequent subset) produced more Gal-9 in the subjects with “detrimental” SNPs (**Figure 2**). There was also a significant increase in Gal-9 production in CD16<sup>pos</sup> non-classical monocytes (data not shown). Together, these data point to functional polymorphisms that result in differential expression of Gal-9 at the gene and protein levels.

### **Minor allelic frequency of *LGALS9* polymorphisms track with ethnic differences in ALD prevalence**

The incidence of ALD and ALD-associated mortality is known to vary according to ethnic origin within racial categories <sup>22</sup>; Hispanic persons have a higher prevalence of chronic alcoholic liver disease than non-Hispanic whites and African-Americans (16.9 vs. 11.1 vs. 9.9 per 100,000, respectively). Moreover, data from a multicenter Veterans Affairs (VA) cooperative study found that among patients with acute alcoholic hepatitis, cirrhosis is more frequent in Hispanic (73%) than in non-Hispanic whites (52%) and African American (44%) patients <sup>23</sup>. Although perhaps confounded by behavioral patterns of alcohol consumption and differences in access to care, age-adjusted mortality rates from alcoholic cirrhosis for Hispanic men is two-fold higher than for non-Hispanic white men <sup>24, 25</sup>.

As our study consisted of European Caucasians, we sought to examine how these SNPs might vary across ethnicities (**Table 3**). Minor allelic frequency (MAF) refers to the frequency at which the least common allele occurs in a given population. A

SNP with an allele (A) frequency of 0.30 (as for rs732222 among Hispanics; **Table 3**) means that 30% of a population has the A allele versus the most common (major) allele, in this case the G allele, which is found in 70% of the population. Using data from the 1000 Genomes Project, we found that the *LGALS9* MAFs (associated with development of ALD) are highest among Hispanics. Thus, these results indicate that racial differences in the relative distribution of *LGALS9* alleles may partially account for the observed ethnic associations with ALD. Of note, the allelic frequencies of our cohort were similar to those reported previously for whites (rs732222: 0.26; rs3751093: 0.23 and rs4239242: 0.38).

## DISCUSSION

This is the first study to examine the potential role of Gal-9 polymorphisms in ALD. We selected Gal-9 as a candidate gene because of recent data implicating roles in a broad range of liver diseases<sup>9-13</sup>. Indeed, we first reported that Gal-9 was produced predominantly by Kupffer cells and induced secretion of pro-inflammatory cytokines<sup>10</sup>. Cytokines have been identified as central effector molecules in multiple steps mediating alcohol-related liver injury, including hepatocyte apoptosis, inflammatory cell recruitment, production of vasoactive molecules, and matrix deposition<sup>26</sup>. In this large series of well-characterized patients with ALD with a control group of 179 subjects who consumed excessive alcohol but remained free of liver disease, we found associations between four *LGALS9* variants and the relative risk of ALD. Data from the UCSC Genome Browser site (hg19) supported a functional role for the majority of the variants associated with development of ALD identified in our study (<http://genome.ucsc.edu/>)<sup>27</sup>. After correction for multiple testing, two of the SNPs were associated with relative risk of developing ALD: rs4239242 ( $p = 0.017$ ) and rs4794976 ( $p = 0.032$ ). Moreover, in vitro, we demonstrated differential induction of Gal-9 at transcriptional and protein expression levels according to these variants. Taken together, these data suggest a genetic basis for the variable expression of Gal-9 that, in turn, modulates the risk of developing ALD, although the precise mechanisms remain undefined. It would also be interesting to determine whether circulating levels of gal-9 in patients who drink excessive alcohol identifies those at risk of developing ALD. Nonetheless, the SNPs identified in the current study have potential utility in the identification and counseling of at-risk individuals. In addition, we also recognize that

Gal-9 displays pleiotropic effects, including regulatory and potentially beneficial properties to limit immunopathology and/or fibrosis that warrant further study <sup>9</sup>.

There are several limitations of the current study worth noting. Although the ALD and control groups were highly similar, including age, gender, race, consumption and duration of alcohol, not all the subjects had liver biopsies. This could have underestimated the presence of mild liver disease in the control group, and hence the number of subjects actually protected from ALD. The study group was highly homogenous (i.e., all European Caucasians and mostly males), potentially limiting the results' applicability. On the other hand, the MAFs reported by the 1000 Genomes Project lead us to speculate that these SNPs might correlate with the ethnic differences in ALD prevalence. Analyses of more diverse cohorts are warranted, as well as patients with less severe forms of ALD (majority in our study had advanced ALD). Finally, in order to determine whether other sequence variations in *LGALS9* contribute to different risks of developing ALD, sequencing of the entire *LGALS9* gene could be considered in an independent cohort.

In summary, Gal-9, a tandem repeat-type galectin with two carbohydrate recognition domains, has been implicated in hepatic immune homeostasis and injury <sup>9-13</sup>. *LGALS9* sequence variations reported here may provide predictive information regarding the risk of developing ALD in at-risk individuals and identify novel therapeutic targets for this common disease.

**ACKNOWLEDGEMENT:**

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**Table 1: Variants in the *LGALS9* gene associated with development of alcoholic liver disease in at-risk individuals.**

SNP*	Genotype	ALD	Acon	Odds Ratio	p Value
<b>RS732222</b>	GG	198 (52.8%)	112 (62.57%)	<b>1.49 (1.04-2.15)</b>	<b>0.035</b>
	GA	159 (42.4%)	57 (31.84%)		
	AA	18 (4.8%)	10 (5.59%)		
RS3763959	GG	112 (30.03%)	52 (32.30%)	0.69 (0.44-1.05)	ns
	GA	192 (51.47%)	69 (42.86%)		
	AA	69 (18.5%)	40 (24.84%)		
<b>RS3751093</b>	GG	217 (56.51%)	123 (66.49%)	<b>1.52 (1.06-2.20)</b>	<b>0.028</b>
	GA	155 (40.36%)	53 (28.65%)		
	AA	12 (3.13%)	9 (4.86%)		
<b>RS4239242</b>	TT	138 (36.51%)	92 (49.73%)	<b>1.72 (1.21-2.46)</b>	<b>0.0034</b>
	TC	194 (51.32%)	74 (40%)		
	CC	46 (12.17%)	19 (10.27%)		
<b>RS4794976</b>	TT	153 (41.13%)	97 (53.01%)	<b>1.66 (1.17-2.34)</b>	<b>0.0065</b>
	TG	196 (52.69%)	67 (36.61%)		
	GG	23 (6.18%)	19 (10.38%)		

\*Abbreviations: SNP Single Nucleotide Polymorphism; ALD Alcoholic Liver Disease; Acon Alcoholic control.

**Table 2: Haplotypes associated with development of alcoholic liver disease in at-risk individuals.**

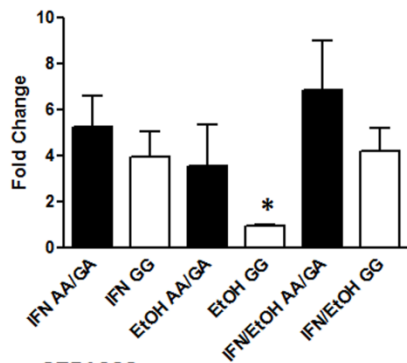
Haplotypes		ALD	Acon	Odds Ratio	p Value
H1	GAGTT	328 (0.41)	168 (0.44)		
H2	AGACG	180 (0.22)	73 (0.44)		
<b>H3</b>	<b>GGGTT</b>	<b>133 (0.17)</b>	<b>86 (0.23)</b>	<b>0.68 (0.5-0.92)</b>	<b>0.016</b>
<b>H4</b>	<b>GGGCT</b>	<b>62 (0.08)</b>	<b>16 (0.04)</b>	<b>1.92 (1.09-3.35)</b>	<b>0.024</b>
H5	GGGTG	28 (0.03)	13 (0.03)		
H6	GAGCG	27 (0.03)	7 (0.02)		
H7	AGGCG	17 (0.02)	13 (0.03)		
	Other	27 (0.03)	4 (0.01)		

**Table 3: Minor allelic frequencies according to ethnicity (see methods and <http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes>).**

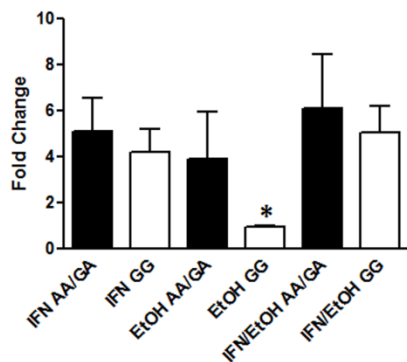
<b>rs number</b>	<b>Hispanics</b>	<b>Europeans-Americans</b>	<b>African-Americans</b>
<b>rs732222 [G]</b>	0.30	0.23	0.196
<b>Rs3751093 [G]</b>	0.267	0.196	0.123
<b>Rs4239242 [T}</b>	0.4187	0.36	0.213

## FIGURES

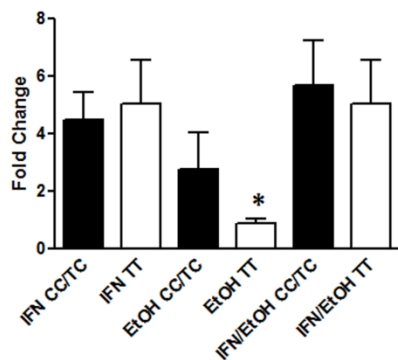
A. rs732222



B. rs3751093



C. rs4239242



**Figure 1: *LGALS9* SNPs are associated with variable gene expression.**

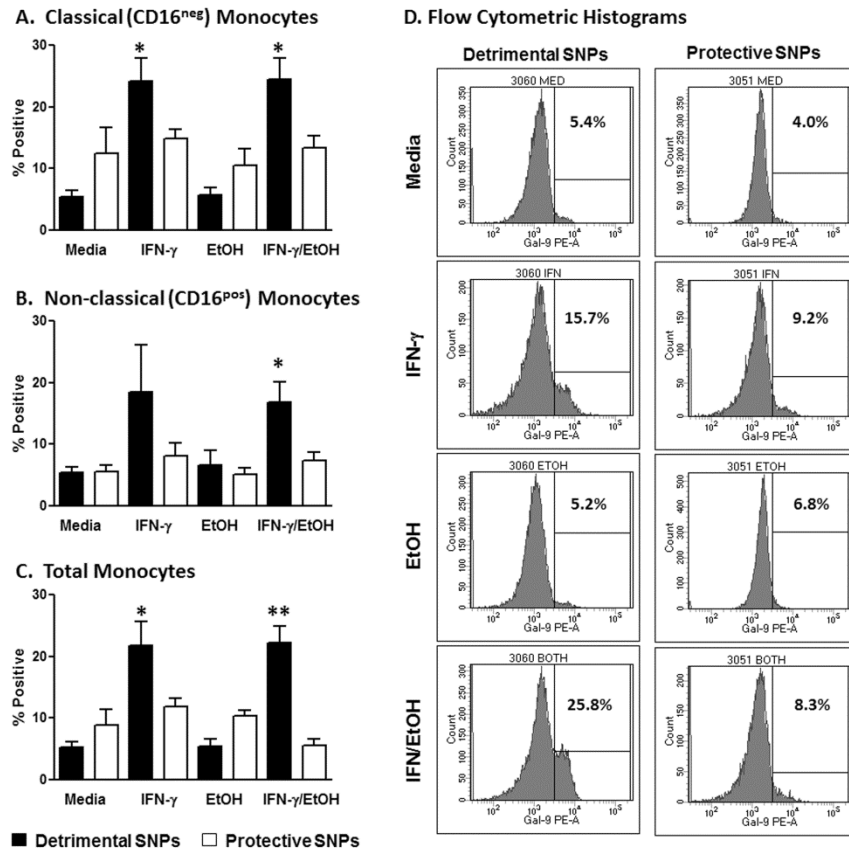
Thirty four normal control individuals were genotyped for SNPs in the *LGALS9* gene.

Peripheral blood mononuclear cells (PBMCs) from these individuals were cultured with

IFN- $\gamma$  (25ng/ml), ethanol (EtOH, 25nM) or a combination of both (IFN/EtOH) for 24

hours. Cells were harvested, RNA isolated, cDNA transcribed and expression of *LGALS9* was assayed using real-time PCR. For the SNPs shown the protective SNP (overrepresented in the control group; white bars) is compared to the other variants (black bars). Fold increase in *LGALS9* expression is shown compared to media alone. Bars represent mean  $\pm$  SEM and significance was tested using Student's T test (\* $p < 0.05$ ).





**Figure 2: *LGALS9* SNPs are associated with variable production of galectin-9.**

We selected 4 individuals who had detrimental SNPs (overrepresented in alcoholic liver disease) and, 4 individuals who had protective (overrepresented in the alcoholic control group) SNPs in the four regions found to be significantly associated with the development of alcoholic liver disease (supplemental Table 1). Peripheral blood mononuclear cells (PBMCs) from these individuals were cultured with IFN- $\gamma$  (25ng/ml), ethanol (EtOH, 25nM) or a combination of both (IFN/EtOH) for 24 hours. Cells were harvested, and cell surface galectin-9 expression (% positive compared to isotype control) by classical (**A**), CD16<sup>+</sup> monocytes and total monocytes (**C**) was determined using flow cytometry. Bars represent mean  $\pm$  SEM and significance was tested using Student's T test (\* $p < 0.05$ ). Representative flow plots of total monocytes treated with a

combination of IFN/EtOH are shown for one individual with detrimental and one with protective SNPs (**C**).

